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9909466.6 23 APR 1999

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Form 1/77 **Patents Act 1977**

❶ Title of invention

1 Please give the title of the invention: Vaccines

❷ Applicant's details

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Country *(and State of incorporation, if appropriate)*: Belgium

2b If you are applying as an individual or one of a partnership please give in full:

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Agent's name MARCUS J W DALTON

Agent's address SmithKline Beecham
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4. Agent's or applicant's reference number *(if applicable)* MJWD/B45182

❺ Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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❼

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8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 [–]

Claim(s) 2 Description 18

Abstract [ ] Drawing(s) [ ]

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MARCUS J W DALTON

Chartered Patent Attorney
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VACCINES

The present invention relates to polysaccharide antigen vaccines, their manufacture and the use of such polysaccharides in medicines. In particular the present invention relates to combined vaccines comprising a polysaccharide antigen, typically a polysaccharide conjugate antigen optionally formulated with either or both a Th1 inducing adjuvant and a protein antigen from Streptococcus Pneumoniae. Such vaccines are particularly useful in the protection of the elderly from Pneumonia.

Streptococcus pneumoniae is a Gram-positive bacteria that is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are 90 known serotypes of pneumococci, and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. Polysaccharides are T-independent antigens, and can not be processed or presented on MHC molecules to interact with T-cells. They can however, stimulate the immune system through an alternate mechanism which involves cross-linking of surface receptors on B cells.

It was shown in several experiments that protection against invasive pneumococci disease is correlated most strongly with antibody specific for the capsule, and the protection is serotype specific.

Polysaccharide antigen based vaccines are well known in the art. Four that have been licensed for human use include the Vi polysaccharide of *Salmonella typhi*, the PRP polysaccharide from *Haemophilus influenzae*, the tetravalent meningococcal vaccine composed of serotypes A, C, W135 and Y, and the 23-Valent pneumococcal vaccine composed of the polysaccharides corresponding to serotypes 1, 2, 3,4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33.

The latter three vaccines confer protection against bacteria causing respiratory infections resulting in severe morbidity and mortality in infants, yet

these vaccines have not been licensed for use in children less than two years of age because they are poorly immunogenic in this age group.

The 23-valent pneumococcal vaccine has shown a wide variation in clinical efficacy, from 0% to 81% (Fedson et al. Arch Intern Med. 154: 2531-2535). The efficacy appears to be related to the risk group that is being immunised, such as the elderly, Hodgkin's disease, splenectomy, sickle cell disease and agammaglobulinemics (Fine et al Arch Intern Med. 154:2666-2677), and also to the disease manifestation. Pneumococcal pneumonia and Otitis media are diseases, which do not have demonstrated protection by the 23-valent vaccine. It is generally accepted that the protective efficacy of the pneumococcal vaccine is more or less related to the concentration of antibody induced upon vaccination; indeed, the 23 polysaccharides were accepted for licensure solely upon the immunogenicity of each component polysaccharide (Ed. Williams et al. New York Academy of Sciences 1995 pp. 241-249).

The present invention provides an improved vaccine particularly for the prevention or amelioration of Pnemococcal infection of the elderly and/or infants.

In the context of the invention a patient is considered elderly if they are 55 years or over in age, typically over 60 years and more generally over 65years. Thus in one embodiment of the invention there is provided a vaccine composition comprising a polysaccharide antigen and a Th1 adjuvant for the prevention of pneumonia in the elderly.

In a second embodiment, the present invention provides a vaccine composition, suitable for use in the elderly and/or Infants comprising at least one *Streptococcus pneumoniae* polysaccharide antigen and one *Streptococcus pneumoniae* protein antigen.

In a third embodiment there is provided a vaccine comprising at least one *Streptococcus pneumoniae* polysaccharide antigen and one *Streptococcus pneumoniae* protein antigen and a Th1 adjuvant.

Typically the *Streptococcus pneumoniae* vaccine of the present invention will comprise polysaccharide conjugate antigens, wherein the

polysaccharide are derived from at least four serotypes. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 11 serotypes are included in the vaccine, for example the vaccine in one embodiment includes the capsular polysaccharide conjugate wherein the polysaccharide are derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F are included. In a preferred embodiment of the invention at least 13 polysaccharide conjugates are included, although more valents, for example 23 valents (such as included in the currently licensed vaccine) are contemplated by the invention.

For elderly vaccination (for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F to the 11 valent vaccine above, whereas for infants serotypes 6A and 19A are advantageously included.

For the prevention/amelioration of pneumoniae in the elderly (+55 years) population and Otitis media in Infants, (typically 18 months to 5 years), it is a preferred embodiment of the invention to combine a multivalent streptococcus pneumonia polysaccharide as herein described with a *Streptococcus pneumoniae* protein or immunologically functional equivalent thereof. Preferred proteins to be included in such a combination, include but are not limited to, pneumolysin (Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2." Mitchell TJ, Mendez F, Paton JC, Andrew PW, Boulnois GJ, Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in Escherichia coli: rapid purification and biological properties." Mitchell TJ, Walker JA, Saunders FK, Andrew PW, Boulnois GJ. WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al) -.WO 99/03884 (NAVA)), PspA and transmembrane deletion variants thereof US 5804193 (Briles et al), PspC (WO 97/09994 - Briles et al). PsaA (Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*." Berry AM, Paton JC). Streptococcal choline binding protein (WO97/41151); Glyceraldehyde – 3- phosphate – dehydrogenase (I&I 64: 3544), HSP 70 (WO96/40928), M like

protein SB patent application No EP 0837130 and adhesin 18627 SB Patent application No. 0834568

The proteins used in the present invention are preferably selected from the group pneumolysin, PsaA, PspA, CbpA (WO97/41151) or a combination of two or more such proteins. The present invention also encompasses immunologically functional equivalents to such proteins, e.g. fragments, deletions such as transmembrane deletion variants thereof, fusions, chemically or genetically detoxified derivatives and the like which are capable of raising substantially the same immune response as the native protein.

Accordingly in an embodiment of the invention there is provided a Streptococcus pneumoniae vaccine comprising a polysaccharide conjugate vaccine comprising polysaccharide antigens derived from at least four serotypes, preferably at least seven serotypes, more preferably at least eleven serotypes and at least one, but preferably two Streptococcus pneumoniae proteins. Preferably one of the proteins is Pneumolysin or PsaA or PspA or CbpA. A preferred combination contains at least Pneumolysin or a derivative thereof and Psp A.

Amongst the problems associated with the polysaccharide approach to vaccination, is the fact that polysaccharides *per se* are poor immunogens. Strategies, which have been designed to overcome this lack of immunogenicity, include the linking of the polysaccharide to large protein carriers, which provide bystander T-cell help. It is preferred that the polysaccharides utilised in the invention are linked to a protein carrier which provide bystander T –cell help. Examples of these highly carriers which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria and Tetanus toxoids (DT, DT crm197 and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD).

A number of problems are, however, associated with each of these commonly used carriers, including both problems in production of GMP constructs and also in immunological characteristics of the constructs. The present invention provides in a preferred embodiment a new carrier for use in the preparation of

polysaccharide -based immunogen constructs, that does not suffer from these disadvantages.

Despite the common use of these carriers and their success in the induction of anti polysaccharide antibody responses they are associated with several drawbacks. For example, it is known that antigen specific immune responses may be suppressed by the presence of pre-existing antibodies directed against the carrier, in this case Tetanus toxin (Di John *et al*; Lancet, December 16, 1989). In the population at large, a very high percentage of people will have pre-existing immunity to both DT and TT as people are routinely vaccinated with these antigens. In the UK for example 95% of children receive the DTP vaccine comprising both DT and TT. Other authors have described the problem of epitope suppression to peptide vaccines in animal models (Sad *et al*, Immunology, 1991; 74:223-227; Schutze *et al*, J. Immunol. 135: 4, 1985; 2319-2322).

In addition, for vaccine which require regular boosting the use of highly immunogenic carriers such as TT and DT are likely to suppress the polysaccharide antibody response after several injections. These multiple vaccinations may also be accompanied by undesirable reactions such as delayed type hyperresponsiveness (DTH).

KLH is known as potent immunogen and has already been used as a carrier for IgE peptides in human clinical trials. However, some adverse reactions (DTH-like reactions or IgE sensitisation) as well as antibody responses against antibody have been observed.

The selection of a carrier protein, therefore, for a polysaccharide based vaccine requires a balance between the necessity to use a carrier working in all patients (broad MHC recognition) and the induction of high levels of anti-polysaccharide antibody responses and of low antibody response against the carrier.

The carriers used previously for polysaccharide based vaccines, therefore have many disadvantages.

The present invention therefore in a preferred embodiment provides a protein D from *Haemophilus influenzae*, or fragments thereof, as a carrier for

polysaccharide based immunogenic composition, such as vaccines. Fragments suitable for use include fragments encompassing T-helper epitopes. In particular protein D fragment will preferably contain the N-terminal 1/3 of the protein.

Protein D is an IgD–binding protein from *Haemophilus influenzae* (EP 0 594 610 B1) and is a potential immunogen.

The vaccines of the present invention are preferably adjuvanted. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

It is preferred that the adjuvant be selected to be a preferential inducer of a TH1 type of response to enable a cell mediated response to be generated. High levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

It is important to remember that the distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of Il-4, IL-5, IL-6, IL-10. Suitable adjuvant systems include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt .

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739.

A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

Preferably the formulation additional comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

Unmethylated CpG containing oligonucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

In a further aspect of the present invention there is provided an immunogen or vaccine as herein described for use in medicine.

The vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts.

In one embodiment there is a method of preventing or ameliorating Pneumoniae in an elderly human comprising administering a safe and effective amount of a vaccine, as described herein, comprising a Streptoccocus Pneumoniae polysaccharide antigen and a Th1 adjuvant, optionally with a streptoccocus pneumoniae protein, to said elderly patient.

In a further embodiment there is provided a method of preventing or ameliorating Otitis media in Infants, comprising administering a safe and effective

amount of a vaccine comprising a Streptococcus Pneumoniae polysaccharide antigen and a Streptococcus pneumoniae protein antigen optionally with a Th1 adjuvant, to said Infant.

Preferably in the methods of the invention as descibed above the polysaccharide antigen is presented as polysaccharide protein conjugate.

The amount of conjugate antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 μg of polysaccharide, preferably 0.1-50 μg, preferably 0.1-10 μg, of which 1 to 5 μg is the most preferable range. The protein content of the vaccine will typically be in the range1 -100μg, preferably 5-50μg, most typically in the range 10 - 25μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

EXAMPLE I

S.pneumoniae capsular polysaccharide:

The 11-valent candidate vaccine includes the capsular polysaccharides serotypes 1,3,4,5,6B, 7F, 9V,14,18C,19F and 23F which were made essentially as described in EP72513.

Each polysaccharide is activated and derivatised using the CDAP chemistry (WO/95/08348) and conjugated to the protein carrier.

All the polysaccharides are conjugated in their native form, except for the serotype 3.Its size was reduced by micro-fluidisation.

Protein carrier:

The protein carrier selected is the recombinant protein D (PD) from Non typeable *Haemophilus influenzae*, expressed in *E.coli*.

EXPRESSION OF PROTEIN D

***Haemophilus influenzae* protein D**

Genetic construction for protein D expression

Starting materials

The Protein D encoding DNA

Protein D is highly conserved among *H. influenzae* of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) I & I *59* : 119-125.

The expression vector pMG1

The expression vector pMG1 is a derivative of pBR322 (Gross et al, 1985) in which bacteriophage λ derived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman et al., 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

The E. coli strain AR58

The E. coli strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil - cI857 ΔH1). N99 and SA500 are E. coli K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

The expression vector pMG 1

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilises signals from lambdaphage DNA to drive the transcription and translation of inserted foreign genes. The vector contains the promoter PL, operator OL and two utilisation sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross

et al., 1985). Vectors containing the PL promoter, are introduced into an *E. coli* lysogenic host to stabilise the plasmid DNA. Lysogenic host strains contain replication-defective lambdaphage DNA integrated into the genome (Shatzman et al., 1983). The chromosomal lambdaphage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981).

The E. coli strain AR58

The AR58 lysogenic *E. coli* strain used for the production of the protein D carrier is a derivative of the standard NIH E. coli K12 strain N99 (F - su - galK2, lacZ - thr -). It contains a defective lysogenic lambdaphage (galE::TN10, lambdaKil - cI857 ?H1). The Kil –phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the lambdaphage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil - cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin resistance in the adjacent galE gene.

Construction of vector pMGMDPPrD

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNSI) was used to construct pMGMDPPrD. The protein D gene was amplified by PCR from the pHIC348 vector (Janson et al. 1991) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between

NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino acids of the NS1 protein followed by the PD protein. This vector was labeled pMGNS1PrD.

Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

-----MDP SSHSSNMANT-----

NS1 Protein D

The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by heat shock at 37°C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant *E. coli* strain is referred to as ECD4.

Expression of protein D is under the control of the lambdaP_L promoter/ O_L Operator.

The host strain AR58 contains a temperature-sensitive cI gene in the genome which blocks expression from lambdaP_L at low temperature by binding to O_L. Once the

temperature is elevated cI is released from O_L and protein D is expressed. At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D is described below:

The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final OD_{650} = 60. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate is clarified by centrifugation and cell debris are removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

Chemistry:

Activation and coupling chemistry:

The activation and coupling conditions are specific for each polysaccharide .These are given in Table 1.

Native polysaccharide (except for PS3) was dissolved in NaCl 2M or in water for injection. The optimal polysaccharide concentration was evaluated for all the serotypes.

From a 100 mg/ml stock solution in acetonitrile ,CDAP (CDAP/PS ratio:0.75 mg/mg PS) was added to the polysaccharide solution.1.5 minute later,0.2M triethylamine was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 2minutes at 25°C.Protein D (the quantity depends on the initial PS/PD ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for 1 hour.

Then, the reaction was quenched with glycine for 30 minutes at 25°C and overnight at 4°C.

The conjugates were purified by gel filtration using a Sephacryl 500HR gel filtration column equilibrated with 0.2M NaCl.

The carbohydrate and protein contents of the eluted fractions were determined .The conjugates were pooled and sterile filtered on a 0.22μm sterilizing membrane. The PS/Protein ratios in the conjugate preparations were determined.

Characterisation:

Each conjugate was characterised and met the specifications described in Table 2.

Polysaccharide and protein content (µg/ml):

The polysaccharide content was measured by the Resorcinol test and the protein content by the Lowry test. The final PS/PD ratio(w/w) is determined by the ratio of the concentrations.

Residual DMAP content (ng/µg PS):

The activation of the polysaccharide with CDAP introduces a cyanate group in the polysaccharide and DMAP (4-dimethylamino-pyridin) is liberated. The residual DMAP content was determined by a specific assay developed and validated at SB.

Free polysaccharide content (%):

The free polysaccharide content on conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with α-PD antibodies and saturated ammonium sulfate, followed by a centrifugation.

An α-PS/α-PS ELISA was used for the quantification of free polysaccharide in the supernatant . The absence of conjugate was also controlled by an α-PD/α-PS ELISA.

Example 2: Pneumolysin was obtained according to the methods of Biochim Biophys Acta 1989 Jan 23;1007(1):67-72 "Expression of the pneumolysin gene in Escherichia coli: rapid purification and biological properties." Mitchell TJ, Walker JA, Saunders FK, Andrew PW, Boulnois GJ.

PspA and transmembrane deletion variants thereof can be obtained according to the methods of US 5804193 (Briles et al).

CbpA can be prepared according to the methods of WO 97/41151.

:

Adjuvant compositions

Protein, either individually or together, from the above examples maybe formulated with the undecavalent Streptoccocus polysaccharide combination of example 1 and as adjuvant, the formulation may comprise a mixture of 3 de -O-acylated monophosphoryl lipid A (3D-MPL) and aluminium hydroxide, or of 3 de -O-acylated monophosphoryl lipid A (3D-MPL) and aluminium phosphate, or 3D-MPL

and/or QS21 optionally in an oil/water emulsion, and optionally formulated with cholesterol, or aluminium salt alone, preferably aluminium phosphate.

3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria *Salmonella minnesota.*

Experiments performed at SmithKline Beecham Biologicals have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral and a TH1 type of cellular immunity.

QS21: is one saponin purified from a crude extract of the bark of the Quillaja Saponaria Molina tree, which has a strong adjuvant activity: it activates both antigen-specific lymphoproliferation and CTLs to several antigens.

Vaccine containing an antigen of the invention containing 3D-MPL and alum may be prepared in analogous manner to that described in WO93/19780 or 92/16231.

Experiments performed at SmithKline Beecham Biologicals have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and TH1 type cellular immune responses. Vaccines containing an antigen such antigens are described in US 5750110.

The oil/water emulsion is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprised 5% squalene 5% tocopherol 0.4% Tween 80 and had an average particle size of 180 nm and is known as SB62 (see WO 95/17210).

Experiments performed at Smith Kline Beecham Biologicals have proven that the adjunction of this O/W emulsion to MPL/QS21 further increases their immunostimulant properties.

Preparation of emulsion SB62 (2 fold concentrate)

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S

microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

Table 1

Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D conjugates

Serotype	1	3 (μfluid.)	4	5	6B	7F
PS conc.(mg/ml)	2.0	3.0	2.0	7.5	5.4	3.0
PS dissolution	NaCl 2M	NaCl 2M	H_2O	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/1	1/1	1/1	1/1	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$pH_a = pH_c = pH_q$	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0

Serotype	9V	14	18C	19F	23F
PS conc.(mg/ml)	2.5	2.5	2.0	4.0	3.3
PS dissolution	NaCl 2M	NaCl 2M	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.75	1/0.75	1/1	1/0.5	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75
$pH_a = pH_c = pH_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	10/9.5/9.0	9.0/9.0/9.0

TABLE 2

Criteria	D01PDJ227	D03PDJ236	D4PDJ228	D5PDJ235	D6PDJ209
Ratio PS/Prot (w/w)	1/0.66	1/1.09	1/0.86	1/0.86	1/0.69
Free polysac. content (%) <10 %	1	1	7	9	0
Free protein content (%) <15 %	8	<1	19	21	9
DMAP content (ng/μg PS) < 0.5 ng/μg PS	0.2	0.6	0.4	1.2	0.3
Molecular size (K_{av})	0.18	0.13	0.12	0.11	0.13
Stability	no shift	no shift	no shift	low shift	no shift

	D07PDJ225	D09PDJ222	D14PDJ202	D18PDJ221	D19PDJ206	D23PDJ212
Ratio PS/Prot (w/w)	1/0.58	1/0.80	1/0.68	1/0.62	1/0.45	1/0.74
Free polysac. content (%) <10 %	1	<1	<1	4	4	0
Free protein content (%) <15 %	8	0.3	3	21	10	12
DMAP content (ng/μg PS) <0.5 ng/μg PS	0.1	0.6	0.3	0.2	0.1	0.9
Molecular size (K_{av})	0.14	0.14	0.17	0.10	0.12	0.12
Stability	no shift	no shift	no shift	no shift	shift	no shift

Claims:

1. A vaccine composition, comprising at least one Streptococcus pneumoniae polysaccharide antigen and one Streptococcus pneumoniae protein antigen or immunologically functional equivalent thereof.

2 A vaccine as claimed in claim 1 wherin the polysaccharide antigen is presented in the form of a polysaccharide–protein carrier conjugate.

3 A vaccine as claimed in claim 2 wherein the carrier protein is selected from the group Diphtheria and Tetanus toxoids (DT and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD).

4 A vaccine as claimed in any of claim 1 to 3 wherein the vaccine comprises at least four polysaccharide antigens from different serotypes.

5 A vaccine as claimed in claim 4 wherein the vaccine comprises polysaccharides from serotypes 6B, 14, 19F and 23F.

6 A vaccine as claimed herein wherein the vaccine comprises at least eleven polysaccharide antigens from different serotypes.

7 A vaccine as claimed herein wherein the vaccine comprises at least thirteen polysaccharide antigens from different serotypes.

8 A vaccine as claimed herein wherein the streptococcus pneumoniae protein or immunologically functional equivalent thereof is selected from the group: pneumolysin , PspA and transmembrane deletion variants thereof, PspC , PsaA , Streptococcal choline binding protein, Glyceraldehyde –3- phosphate – dehydrogenase and CbpA.

9 A vaccine as claimed herein comprising at least two Streptococcus pneumoniae proteins or immunologically functional equivalents thereof.

10 A vaccine as claimed herein additionally comprising an adjuvant.

11 A vaccine as claimed in claim 10, wherein the adjuvant comprises an aluminium salt.

12 A vaccine as claimed in claim 10, wherein the adjuvant is a preferential inducer of a TH1 response.

13 A vaccine as claimed in claim 10 or 12, wherein the adjuvant comprises at least one of the following: Monophosphoryl Lipid A or derivative thereof, a saponin immunostimulant, an immunostimulatory CpG oligonucleotide.

14 A vaccine as claimed in claim 13 wherein the adjuvant comprises a carrier selected from the group comprising an oil in water emulsion, liposome and aluminium salt.

15 A vaccine composition as claimed herein for use as a medicament.

16 A method of preventing or ameliorating Streptoccocus Pneumoniae infection in a patient over 55 years, comprising adminstering an effective amount of a vaccine comprising a streptococcus Pneumoniae polysaccharide and either a TH1 inducing adjuvant or a Streptococcus Pneumoniae protein or both.

17 Use of a Streptoccocus pneumoniae protein antigen and/or a Th1 inducing adjuvant and polysaccharide antigen in combination, in the manufacture of a medicament for the prevention of pneumonia in patients over 55 years.

18 A method of making a vaccine as claimed herein, comprising the step of mixing the polysaccharide antigen and the protein antigen.

19. A method of preventing or ameliorating Otitis media in Infants, comprising administering a safe and effective amount of a vaccine comprising a Streptococcus Pneumoniae polysaccharide antigen and a Streptococcus pneumoniae protein antigen optionally with a Th1 adjuvant, to said Infant.